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Effects of long-term exposure to oxytetracycline on phytoremediation of swine wastewater via duckweed systems

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ABSTRACT

The effects of antibiotics on phytoremediation systems have attracted widespread attention to high concentrations of antibiotics in livestock wastewater. In this work, the effects of oxytetracycline (OTC) whose concentration was 0.05–1.00 mg/L on swine wastewater treatment by a duckweed-based phytoremediation systems were explored, including oxidative stress, nutrient production, bioconcentration, and community-level physiological profile. Results showed that the levels of H_2O_2 and peroxidases (PODs) of duckweed increased with an increase of OTC in the first 8 days. However, oxidative stress of duckweed disappeared after 18 days of exposure, except for 0.05 and 1.00 mg/L. Although OTC has negative effects on the production of high-value nutrients in duckweed, 0.05 and 0.25 mg/L OTC promoted the synthesis of starches and flavonoids, and the synthesis of vitamin C could restore after 28 days of exposure. In addition, a community-level physiological profile revealed that 0.05 mg/L OTC could significantly enhance the duckweed associated microorganisms metabolic activity. Therefore, this investigation adds to the understanding of antibiotics stress on high-value nutrients production in hydrophyte when was used to livestock wastewater management and also helps to clarify the metabolism profile of the phyllosphere and rhizosphere microbes; thereby providing new insight into effects of antibiotic on livestock wastewater phytoremediation.

1. Introduction

A large number of livestock farms have been continuously established and expanded to satisfy the increasing requirement for high-quality proteins and meat due to the rapid growth of the global economy and population. For instance, the production of poultry meat, beef, pork, mutton, poultry eggs, and milk was increased by 10.2, 11.9, 3.1, 7.1, 5.4, and 13.0 times from 1996 to 2015 in China, and resulted in the fast increased of livestock farms (Fig. S1)(Hu et al., 2017). Subsequently, livestock wastewater, including swine wastewater containing high concentrations of phosphorus and ammonium discharged from livestock farms is dramatic increases, which has risen the concerns in public (Li et al., 2020b, 2018a). In addition, to advance livestock growth, development, and prevent microbial infections, incomputable antibiotics are extensively added to animal fodder, which contributed to the occurrence of antibiotics in swine wastewater and water bodies incrementally (Hu et al., 2019b; Keerthisinghe et al., 2019; Lin et al., 2019, 2020; Wu et al., 2018; Zhou et al., 2020; Zubair et al., 2020). According to the report by Cheng et al. (2020), the most continually detected antibiotics in swine wastewater are sulfonamides and tetracyclines, with the levels up to 324.40 and 685.60 μg/L, respectively. Antibiotics not only inhibit the activity of microorganisms in the treatment systems but also promote the enhancement of bacterial resistance since antibiotics have higher biological activity, which has attracted widespread attention (Cheng et al., 2020; Lou et al., 2018; Weber et al., 2011).

Oxytetracycline (OTC), a widely used tetracycline antibiotics, is designed to particularly combine to the A position of the ribosome 30 S subunit, preventing the coupling of aminoacyl-tRNA at this position,

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thereby limiting the development of the peptide chain then affecting proteins synthesis. However, because it is difficult to completely uptake and metabolize by alimentary canal, OTC often presents in swine wastewater and water bodies with high concentration (maximum to 993.8 μg/L), so to some extent, it often causes unexpected effects on organisms (Hu et al., 2019b; Ma et al., 2016; Santaeufemia et al., 2016; Zhu et al., 2020; Li et al., 2018b). Consequently, how to effectively address this pollutant has attracted more and more attention.

Phytoremediation, an eco-friendly, low-cost, and sustainable method is an extensively and efficient applied environmental cleanup biotechnology on the basis of phytoextraction, rhizofiltration, phytostabilization, phytodegradation, and phytovolatilization by plant and associated microorganisms (Hu et al., 2020; Zhou et al., 2018). Phytoremediation is universally applied in inorganic and organic pollutant treatment in the environment due to the high-efficiency (Pilon-Smits, 2005; Pant and Adholeya, 2007, 2009; Shao et al., 2020). Among various aquatic plants, duckweed is widely employed due to its fast growth rate, high nutrients content, easy to harvest, and can be used to produce energy (Li et al., 2020a; Zhou et al., 2019). Li et al. (2017) illustrated that the removal rate of paracetamol, caffeine, and triclosan could up to 100% via *Spirodela polyrhiza* treatment systems, and 90% COD, 83.3% ammonium, and 80.8% phosphorus could also be removed simultaneously. Nevertheless, although there are several kinds of researches using duckweed as a model to investigate the toxic effects of antibiotics on aquatic organisms, the literatures about ecotoxicological risks of antibiotics to duckweed when duckweed is applied in livestock wastewater treatment are still infrequent, and the biological effects mechanisms need to be further clarified (Gomes et al., 2017; Hu et al., 2020; Iatrou et al., 2017).

Microorganisms play an important role in a variety of biotechnology process (Park and Liang, 2016). The development, carbon sequestration, and reproduction of plants can partly attribute to the microflora that harbored on the rhizosphere of vegetation (Lundberg et al., 2012). For example, the removal of nitrogen, phosphorus, and other pollutants by rhizosphere microorganisms played an important role during the phytoremediation of swine wastewater via duckweed. It has been reported that more than 23% NH_3-N could be removed from duckweed ponds by nitrification of rhizosphere microorganisms (van der Steen et al., 1999). Yamaga et al. (2010) also found that the growth of duckweed in naturally systems could be significantly affected if microorganisms coexist with that. Therefore, it is vital to understand how antibiotics affect the phyllosphere and rhizosphere microbes (RPM), and then oppress the removal of contaminants. However, knowledge about the change of the metabolism profile of microbes at the presence of antibiotics, as well as the removal of antibiotics by floating macrophytes systems which applied to livestock wastewater management, is still piecemeal.

Therefore, in the present work, the oxidative stress response, nutriments levels, and bioconcentration of duckweed under different concentrations of OTC (0.05, 0.10, 0.25, 0.50, and 1.00 mg/L) were investigated to assess the tolerance and recycle risks of duckweed. In addition, what is more important is that the community-level physiological profile of duckweed RPM was explored through biolog ECO-plate to better understands the mechanisms of antibiotics on phytoremediation systems in microscopically. This is the first work to evaluate the ecological risks of OTC for livestock wastewater phytoremediation by the duckweed systems in microscopically. The significance of this work is to clarify the effects of antibiotics on hydrophyte and also help to illustrate the mechanisms of antibiotic-microbes interaction, which to the benefit of better construction of livestock wastewater phytoremediation systems.

2. Materials and methods

2.1. Materials and characteristics

2.1.1. Swine wastewater

The wastewater applied in this work for batch tests was synthetic wastewater according to our previous work, and the pH value of the wastewater was maintained at 6.5 ± 0.5 by 0.1 mol/L NaOH solution (Hu et al., 2019b). The swine wastewater was disinfected in a high-pressure steam sterilizer (YX-280, Hefei Huatai Medical Equipment Co., Ltd., Hefei, China) for 30 min under 121 ℃ before the study, and the ionic concentration of the substrate was listed in Table S1.

2.1.2. Duckweed

The species of the duckweed applied in this work was *Lemna aequinoctialis*, which was obtained from a constructed freshwater pool, Changsha, Hunan, China. In order to shield the influence of wild environment on duckweed, obtained duckweed was stabilized in the laboratory for 2–4 weeks.

2.2. Experimental methods

The ultrapure water was used to wash duckweed after harvest, and then the fresh weight was measured with the balance rapidly after the water was sucked on the filter paper. The oxidative stress response and nutrients levels of L. *aequinoctialis* were measured via destructive sampling via taking a whole beaker as a sample. The pH meter (PHS-3C, Shanghai INESA Scientific Instrument Equipment Co., Ltd., Shanghai, China) was used to monitor the pH values of synthetic swine wastewater to evaluate the growth condition of L. *aequinoctialis*.

2.2.1. Duckweed cultivation methods

Before the study, the growing vigorously L. *aequinoctialis* was chosen and rinsed with ultrapure water and then placed into the 500 mL beaker contained 400 mL swine wastewater and the same weight of *Lemna* (0.3 ± 0.03 g fresh weight) was seeded to overspread the 60–80% water surface. And then cultured in a constant temperature light incubator (GZX-450, Beijing Zhongxingweiye Instrument Equipment Co., Ltd., Beijing, China), and the culture conditions were described as previously by Hu et al. (2019b). Except for the control beakers, various OTC concentrations substrate (0.05, 0.10, 0.25, 0.50, and 1.00 mg/L) was achieved through added different volumes OTC solution into simulate swine wastewater. Since the pH of the swine wastewater would decrease during the cultivation process, the pH was detected and maintained at 6.5 ± 0.5 through 0.1 mol/L NaOH. The experiments were conducted in triplicates.

2.2.2. Concentrations of H_2O_2 and PODs in L. aequinoctialis

The guaiacol method was used to determine the PODs levels in duckweed. Originally, got 0.2 g of washed fresh duckweed into a homogenizer, and 0.05 mol/L phosphate buffer solution (PBS, 1.6 mL) was added then homogenize under ice-water bath conditions. The supernatant was the PODs enzyme solution after the obtained homogenate was centrifuged at 4 ℃ and 12,000*g* for 20 min. The 3 mL of the reaction solution was added into the enzyme solution (30 μL). Adjust zero with PBS solution as a control, and measure the absorbance at 470 nm for 40 s (T6 series, Beijing Purkinje General Instrument Co., Ltd., Beijing, China). Calculate PODs contents according to Eq. 1:

$$PODs = (\Delta 470 \times V_t)/(V_s \times W \times t \times 0.01) \quad (1)$$

Where Δ470 represented the change of absorbance during the reaction time, t represented the reaction time (40 s), respectively, V_t represented the total volume of the extracted enzyme solution, V_s represented the total volume of the enzyme solution used in the measurement, and W represented the fresh weight of duckweed.

Taken a certain amount of fresh duckweed and added 9 times the volume of normal saline to homogenize under the ice-water conditions for H_2O_2 measurement. The obtained homogenate was centrifuged at 10,000 rpm for 10 min and measured via a hydrogen peroxide kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.2.3. Determination of vitamin C, starch and flavonoid of L. aequinoctialis

Vitamin C has an extremely high nutritional value and is abundant in duckweed. Obtained a certain amount of fresh duckweed and added 2% oxalic acid (3 mL) homogenized under ice-water bath conditions. Then, the homogenate was poured into a 100 mL volumetric flask. Meanwhile, 15% potassium ferrocyanide (1 mL) and 30% zinc sulfate (1 mL) was added, and then made up to 100 mL with 1% oxalic acid solution and filtered. Adding 2 mL of staining solution and 5 mL of xylene in order into the filtrate (4 mL), and measure the absorbance at 500 nm. Starches and flavonoids were determined through the plant starches content determination kit and a plant flavonoids detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively.

2.2.4. OTC concentration in L. aequinoctialis

The OTC concentration in L. *aequinoctialis* was done using the modified procedure of Ahmed et al. (2015). In detail, 50 mg freeze-dried and crushed duckweed was extracted with 0.2 M citrate buffer with pH = 4.7 adjusted with sodium hydroxide, mix for 1 min with a vortex mixer. After that, the extraction was sonicated (10 min) and centrifuged (4 000 rpm, 10 min). This process was repeated 3 times, and the supernatant was combined. Next, the samples were washed repeatedly with 10 mL of n-hexane, and the upper n-hexane was removed after standing. The SPE cleanup procedure was performed by OASIS HLB 60 mg cartridge, and the extraction was detected by HPLC-MS.

2.2.5. Community-level physiological profile

Community-level physiological profile (CLPP) of RPM samples was investigated followed the method described by Zhao et al. (2018). Firstly, 20 pieces of harvested duckweed were rinsed with sterile water gently, and then the duckweed was transferred to a centrifuge tube (50 mL) contained sterile water (15 mL). After that, duckweed was sonicated for 5 s at 300 W for 6 cycles to disperse the associated microorganisms. The suspension was injected to the biolog ECO-plates with a pipette in 150 μL and incubated in an incubator at 25 ℃ for 7 days. Then, the plates were measured every 12 h by ELIASA at an absorbance of 590 nm.

The absorbance value of all samples (96 h) was classified as the key point for RPM carbon metabolic function analysis. The average well color development (AWCD), representing the average metabolic activity over all wells and per value, is calculated as:

$$AWCD = \frac{1}{31}\sum_{i=1}^{31}\left(A_i - A_0\right) \quad (2)$$

where A_0 represented the absorbance value of the blank well and A_i was the absorbance value of well i. The relative value of wells may be negative, in which cases they were set to zero during the analysis.

2.2.6. Statistical analysis

All data were compared via One-way ANOVA. SPSS 17.0 (SPSS, Chicago, IL, USA) was utilized to conduct statistical analyses, with significance level $p < 0.05$. Principle component analysis (PCA) was performed by using Origin 2017 (OriginLab Corporation, USA) to investigate the differences in the utilization of carbon sources by duckweed RPM under various OTC concentrations. The results of the work were expressed as mean ± SE (standard error) of the three replicates. Other figures in this work were all constructed by Origin 2017.

3. Results and discussion

3.1. Effects of OTC on H_2O_2 and PODs levels of L. aequinoctialis

3.1.1. H_2O_2

ROS, such as superoxide anion (·O2ˉ), hydroxyl radical (•OH), and hydrogen peroxide (H_2O_2) can facilitate a series of significant biological processes in plants including development, reproduction, immune defense, stress acclimation and seed germination (Apel and Hirt, 2004; Dowling and Simmons, 2009). It was indicated by Fig. 1a that the concentration of H_2O_2 in L. *aequinoctialis* was remarkably affected by OTC. Firstly, high-level OTC could enhance the synthesis of H_2O_2 in duckweed compared with that of the control group on day 4. The H_2O_2 concentrations in treatment groups were 19.15%, 10.64%, 6.38%, 8.51%, and 29.79% higher than those in the control, respectively. Secondly, the H_2O_2 concentrations of all treatment groups reached maximum on day 8. The highest H_2O_2 level in the 1.00 mg/L treatment group was 0.34 mmol/g, and that of the control group was 53.27% lower than that ($p < 0.05$). That means that promoting the production of ROS could be an effective method for duckweed to alleviate the toxic effect caused by antibiotics when it was used for phytoremediation of swine wastewater which was polluted by antibiotics. Finally, the effects of ROS are dose-dependent, excessive doses could quickly attack chloroplasts, nucleic acids, proteins and even destroy the ROS scavenging systems, which in turn could cause oxidative damage to occur in plant cells (Apel and Hirt, 2004; Cakmak, 2000). It was observed in our previous work that OTC caused a decrease in chlorophyll and xanthophyll contents in duckweed cells (Hu et al., 2019b).

In contrast, the synthesis of H_2O_2 shown different trends compared with the first 8 days with the extension of cultivation. On the one hand, the concentrations of H_2O_2 in duckweed with the low-concentrations treatment (0.05, 0.10, and 0.25 mg/L) were similar to those in the

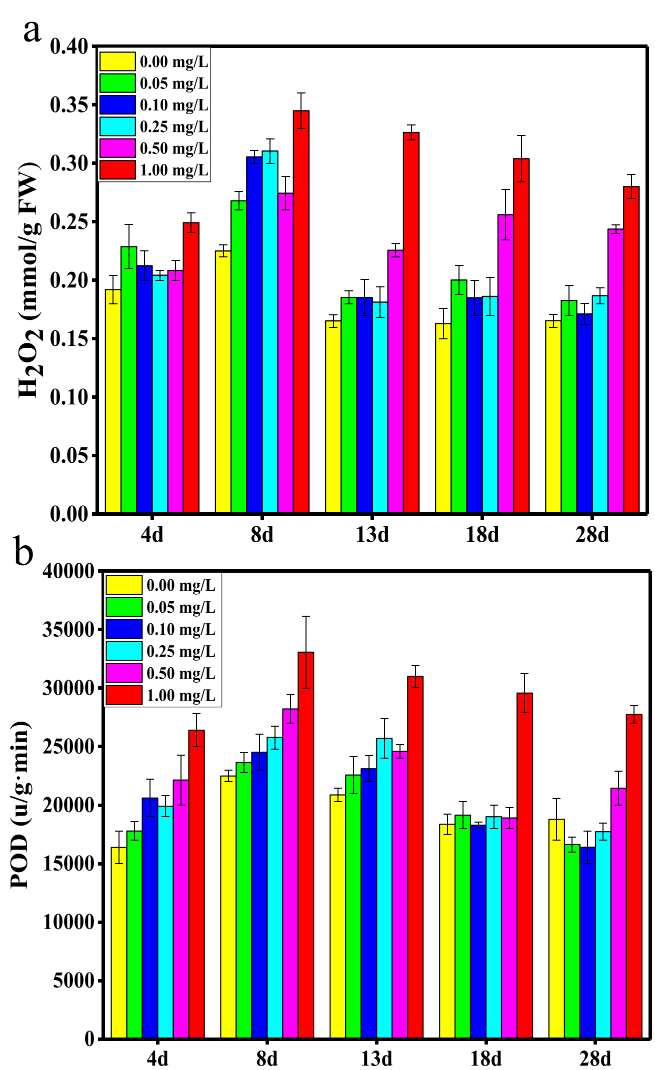

Fig. 1. Contents of H_2O_2 and PODs in duckweed within 28 days. (a) H_2O_2; (b) PODs.

control group. On the other hand, the high-concentration treatment (0.50 and 1.00 mg/L) still affected significantly on the production of H_2O_2 in duckweed ($p < 0.01$). In this context, the duckweed could accommodate to low concentrations of antibiotic after prolonged exposure to antibiotics. The toxicity was relieved, and the intracellular ROS content was reduced. This is consistent with the phenomenon of duckweed leaves changed from light yellow to green during the investigation observed by the authors. Therefore, the results of this work showed that stress of OTC on *Lemna* could be relieved if the antibiotics concentration in livestock wastewater are lower, which favors the phytoremediation of livestock wastewater by macrophyte systems (Fig. 1a).

3.1.2. PODs

PODs is a major member of the antioxidant defense system, which can defend the harmful effects of ROS and decompose H_2O_2 into water and oxygen (Mishra et al., 2006). Changes in the activity of duckweed antioxidant enzymes can describe the dissipation efficiency of intracellular ROS (Song et al., 2012). As can be seen from Fig. 1b, the PODs activity of L. *aequinoctialis* in the treatment with 1.00 mg/L OTC increased by 61.25%, 47.08%, 48.55%, 61.07% and 47.79% ($p < 0.05$) respectively, compared with those of the control. This indicated that the high level of OTC could significantly enhance the production of PODs in duckweed, and caused severe oxidant stress. Zhu et al. (2020) founded similar results when investigated the ecotoxicological effects of sulfonamide on the submerged plant *Vallisneria natans* (Lour.) Hara. In contrast, a recent study reported that sulfamethoxazole and norfloxacin could contribute to a decrease in PODs activity in *Chlorella* sp. (Niu et al., 2019). The types of antibiotics, plant species and culture conditions may be the reasons for the divergence between the above reports and the results of this work.

In addition, the effects of OTC at other concentrations (0.05, 0.10, 0.25, and 0.50 mg/L) on the production of PODs in duckweed showed a similar trend to H_2O_2, following the dose-response relationships. Meanwhile, the PODs contents in hydrophyte at the low levels (0.05, 0.10, and 0.25 mg/L OTC) were the same as that of the control group as the cultivation progresses. The results described that the oxidative stress of duckweed caused by low concentrations of OTC could be renovated by drawn-out exposure, which is beneficial to the growth of duckweed in antibiotics contaminated swine wastewater.

3.2. Effects of OTC on vitamin C, starches and flavonoids synthesis

3.2.1. Vitamin C

As a common nutrient, vitamin C is rich in *Lemna* L. The fluctuation of vitamin C contents in duckweed under different original OTC levels was revealed in Fig. 2a. Obviously, OTC could significantly inhibit the production of vitamin C in L. *aequinoctialis* cells on day 4. The vitamin C concentrations in the treatment groups were 20.34%, 26.89%, 43.84%, 33.76%, and 37.91% lower compared with those in the control group, respectively ($p < 0.01$). This could be attributed to the production of critical enzymes such as L-Gulose lactone oxidase and D-Galacturonic acid reductase which taking part in the synthesis pathways of vitamin C was inhibited since the ROS-inducing protein carbonylation (an irreversible oxidative process), and then the decreases of the vitamin was visible when the hydrophyte was exposed to the swine wastewater which contaminated by OTC (Romero-Puertas et al., 2002). Previous studies have also shown that vitamin C levels would decrease when duckweed was subjected to adverse environmental stresses (Karatas et al., 2009). In the meantime, vitamin C has the ability to remove singlet oxygen. The decrease of vitamin C caused by OTC also shown that the excessively free radicals were generated under stress, and then damaged duckweed structures which associate with vitamin C production. Therefore, the organelles such as chloroplast could be damaged via interference with the steady-state control and energy conduction of ROS (Foyer and Lelandais, 1996; Isidori et al., 2005).

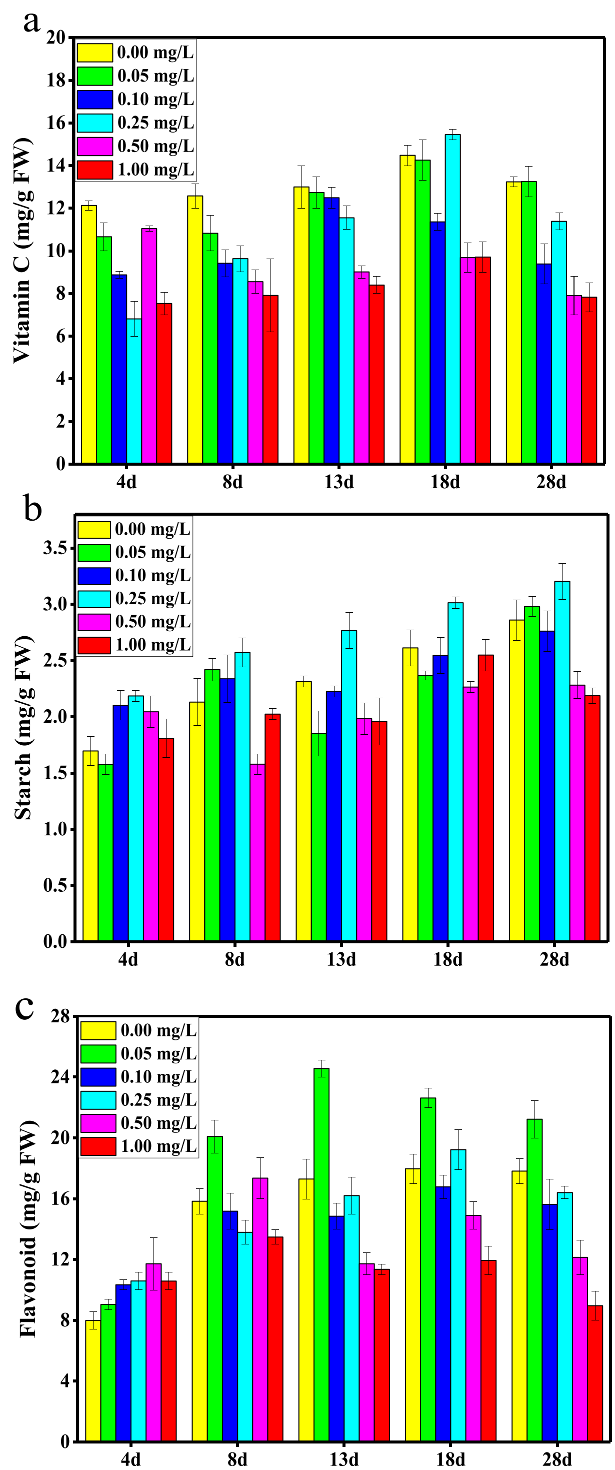

Fig. 2. The dynamic changes of vitamin C, starches and flavonoids levels in duckweed within 28 days. (a) Vitamin C; (b) Starches; (c) Flavonoids.

The vitamin C production at 0.05 mg/L OTC gradually increased with the culture from 8 to 28 days, and it was almost equal to that of the control group on day 28. This demonstrated that the physiological damage caused by low concentration antibiotics to duckweed could be remediated. Although it has taken a long time, the physiological function after recovery was no different from that of the control group. Nevertheless, 0.10, 0.25, 0.50, and 1.00 mg/L OTC still significantly inhibited the yield of vitamin C, which showed that the damage caused by OTC may exist permanently. However, previous research by Hu et al. (2019a) showed that the concentration of vitamin C in *Lemna minor* L. cells would increase under cobalt stress, which may be caused by different mechanisms of toxic effects of heavy metals and antibiotics. The results of this investigation indicated that the presence of low levels of antibiotics in swine wastewater was not restricted to recycle high-value vitamins from duckweed while using duckweed for phytoremediation of swine wastewater.

3.2.2. Starches

The starches level of duckweed could reach 70% of the dry weight (Cui and Cheng, 2015). Therefore, duckweed can be used as the raw materials for the production of biofuels and animal feeds, which endows a great significance for exploring the changes of starches in duckweed under antibiotics stress. Changes in starches content in duckweed caused by OTC stress were shown in Fig. 2b. Distinctly, when duckweed was exposed to OTC, the treatment groups significantly stimulated the production of starches in duckweed to some extent due to hormesis ($p < 0.01$). The maximum increase in starches content was 32.27% compared with that of the control group. The phenomenon illustrated that antibiotic-induced plant stress promoted the accumulation of starches in plants, and Taiz and Zeiger (2010) reported that this is part of the defense systems of physiological stress caused by abiotic and biotic factors.

Dissimilarly, the following detection results indicated that 0.25 mg/L OTC could remarkably promote the generation of starches in L. *aequinoctialis*. At different time periods, the starches content of 0.25 mg/L OTC treatment group increased by 22.38%, 25.80%, 24.54%, 25.66% and 15.94%, respectively, compared to those in the control group ($p < 0.05$). It was assumed that in this case, OTC could facilitate a series of energy and materials transfer of duckweed photosynthetic systems and would not be affected by ROS, thereby promoted the accumulation of starches in duckweed. In addition, OTC stress suppressed the growth of plants, the photosynthetic molecular carbohydrates were not utilized in growth-related metabolisms, and starches began to accumulate in duckweed leaves immediately (Sree and Appenroth, 2014). Ultimately, duckweed is weakly stressed by low concentration of OTC (0.05 and 0.10 mg/L), which not only couldn't stimulate the photosynthetic systems to make changes but also damaged by ROS simultaneously, resulted in inhibition of starches synthesis. In addition, high concentrations of OTC (0.50 and 1.00 mg/L) have strong inhibitory effects on chloroplast function and reduced export of photosynthate from the plastids, which limited the production of starches (Hu et al., 2019b).

3.2.3. Flavonoids

A variety of natural substances in plants can be used for anti-inflammatory and antibacterial (Chinnappan et al., 2018, Muthusamy et al., 2017). For example, *Lemna* L. is rich in flavonoids, and this kind of plant secondary metabolites can be used for anti-inflammatory, antibacterial, and scavenge free radicals from the body (Friedman, 2014, Huang et al., 2014). However, the effects of antibiotics stress on flavonoid synthesis in duckweed have not been studied. Therefore, changes in the flavonoids level of L. *aequinoctialis* under long-term OTC stress were shown in Fig. 2c. Above all, the initial antibiotic exposure could significantly elevate the augment of flavonoids capacity in duckweed. Different from the unstressed duckweed, the concentrations of flavonoids in duckweed cells increased by 38.21%, 29.45%, 32.60%, 46.87%, and 32.52%, respectively, under OTC stress ($p < 0.01$). Huang et al. (2014) declared that starvation could significantly promote the expression of enzymes involved in flavonoid biosynthesis in protein levels. Therefore, they assumed that stresses could affect the synthesis of flavonoids by changing the activities of key enzymes, which was consistent with the results of this work. In addition, observed that the contents of H_2O_2 and PODs in duckweed were also boosted during the same period (Fig. 1), which demonstrated that flavonoids may also participate in the process of scavenging free radicals (Akhtar et al., 2010). Additionally, the flavonoids concentration of L. *aequinoctialis* was not affected by low-concentration OTC (0.05 mg/L) stress and the concentration was higher than that of the control group at different detection periods, with a maximum of 39.50% (on day 8).

Therefore, in the treatment of swine wastewater with duckweed systems, the stress of antibiotics on L. *aequinoctialis* could be alleviated by reducing the concentrations of antibiotics in the inflow, and would not influence the recovery and extraction of high-value nutrients such as flavonoids from duckweed.

3.3. OTC removal kinetics and OTC concentrations in duckweed

3.3.1. OTC removal kinetics analysis

In the present work, the Michaelis-Menten kinetic model, the Modified logistic kinetic model, the Second-order kinetic model, and the First-order kinetic model (Supplementary Material) was applied to fit the removal of OTC by the duckweed systems. The fitting results parameters for different concentrations of OTC were exhibited in Table 1, and the fitting plots were shown in Fig. 3. Obviously, although the removal efficiency of phytoremediation was lower than other treatment approaches, such as electrocoagulation and photocatalysis, the concentration of OTC continuously decreased with time prolonged (Zaied et al., 2020; Lin et al., 2019). However, the removal efficiencies of various concentrations of OTC presented significant differences after treatment by the duckweed systems within 10 days. The highest OTC removal rate (0.50 mg/L, 96.79%) was 26% higher than the lowest one (0.05 mg/L, 70.80%). Trapp and Matthies (1995) suggested that the ingestion of antibiotics and other organic chemicals by plants is mediated by passive diffusion transport. This may be the reasons that the removal efficiency of high concentration OTC through the duckweed systems was higher than that of low concentration.

The Michaelis-Menten kinetic model had a higher R^2 value compared with the other three models, on OTC removal via the duckweed systems. In this context, the Michaelis-Menten kinetic model could better illustrate the removal of different levels of OTC by duckweed. In addition, the maximum specific growth (k_1) shown different trends, the value of k_1 increased with the improvement of initial OTC concentration in the swine wastewater. Half saturation constant (k_2) is an important parameter which referred to as the affinity constant of the substrate, where, the compounds with low k_2 values indicate higher affinity, while, those with higher k_2 indicate low affinity or inefficiency of the vegetation for biodegradation (Kong et al., 2018, Mulder and Hendriks, 2014). As shown in Table 1, compared with other groups, the k_2 was highest when the concentration of OTC was 1.00 mg/L. This result indicated that high levels of xenobiotics inhibited the biodegradation of OTC, which may be due to the high concentrations of OTC limited the growth of and related physiological activities associated with antibiotic metabolism in duckweed (Gomes et al., 2017; Hu et al., 2019b). However, the k_2 value of some experimental groups was negative. Although some scholars believe that the negative value of k_2 was no physical implication, Converti et al. (1999) regarded the negative value of k_2 as the maximum degradation rate of pollutants (R.W. et al., 1969). Meanwhile, the value of r_f of the First-order kinetic model gradually increased as the increases of OTC concentration (0.05–0.50 mg/L) in the medium also support this opinion. Similar trends for antibiotic degradation by aquatic plants were illustrated in a previous study (Kiki et al., 2020).

3.3.2. OTC concentrations in duckweed and BCF

The content of OTC in the plant tissue increased continuously with the increased of initial OTC concentration in the swine wastewater (Fig. 4). Initially, at the end of the 10-day cultivation, the OTC level in the L. *aequinoctialis* ranged from 30.62 to 93.88 μg/g DW. It was higher than Gomes et al. (2017) reported that around 60 μg/g DW of ciprofloxacin in duckweed after cultivation. Although there is no standard to limit the concentrations of antibiotics in aquatic plants, the higher antibiotics concentrations (whether it is fresh or dry) in the body are not

Table 1
The important parameters and statistics indexes of the models for OTC removal.

OTC	First-order kinetic model		Second-order kinetic model		Michaelis-Menten kinetic model			Modified logistic kinetic model		
mg/L	r_f	R_f^2	r_s	R_s^2	k_1	K_2	R_m^2	r_l	a	R_l^2
0.05	0.1206	0.9987	3.9554	0.9920	0.0035	-7.0631 × 10–4	1.0000	0.1043	-0.3120	0.9840
0.10	0.1710	0.9832	4.5697	0.9842	0.0079	-0.0037	0.9968	0.2465	0.4316	0.9967
0.25	0.2178	0.9376	5.2983	0.9878	0.0263	-0.0029	0.9988	0.4163	0.7268	0.9967
0.50	0.2396	0.8867	4.6400	0.9893	0.0738	0.0511	0.9996	0.5121	0.8354	0.9964
1.00	0.1888	0.9480	0.8409	0.9861	0.1668	0.3026	0.9997	0.3672	0.7039	0.9956

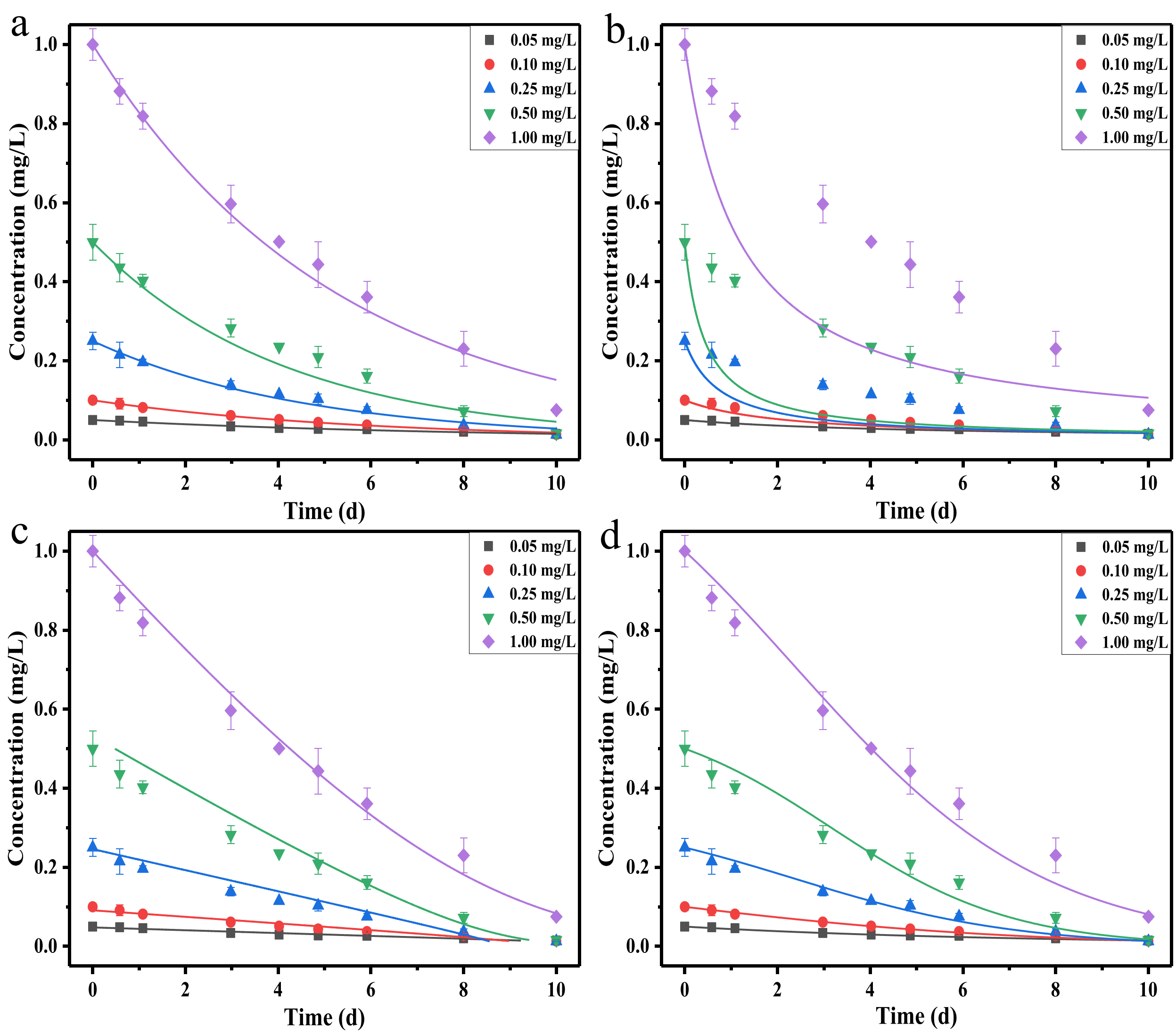

Fig. 3. The kinetic models for various concentrations of OTC removal by duckweed systems.(a) First-order kinetic model; (b) Second-order kinetic model; (c) Michaelis-Menten kinetic model; (d) Modified logistic kinetic model.

conducive to the utilization of duckweed which was used for phytoremediation of livestock wastewater.

Bioconcentration factor (BCF), a dimensionless ratio of the level of xenobiotics in the organic to that in the medium, which is a significant index employed to evaluate the ability to accumulate foreign chemical compounds from the external environment (Neely, 1981). As indicated by Fig. 4, different from the trend of OTC concentration in duckweed, the highest BCF value (0.61) was attained when the initial OTC concentration was 0.05 mg/L. With the increases of OTC in swine wastewater, the value of BCF decreased from 0.61 to 0.09. The reduction of BCF could result from metabolic changes upon exposure to OTC that affected the ability of *Lemna* to uptake the antibiotic. It has indicated that antibiotics can be consumed, transported, and metabolized via glycosylation and glutathione pathways in hydrophytes and proteins are widely involved in these processes (Liu et al., 2013). In our previous research, the decrease of protein content in duckweed was illustrated when the OTC concentration was higher (>0.25 mg/L) (Hu et al., 2019b). Finally, high levels of antibiotics could also increase the production of H_2O_2 in *Lemna* (Fig. 1), which caused the duckweed to undergo severe lipid peroxidation and induced serious damage to

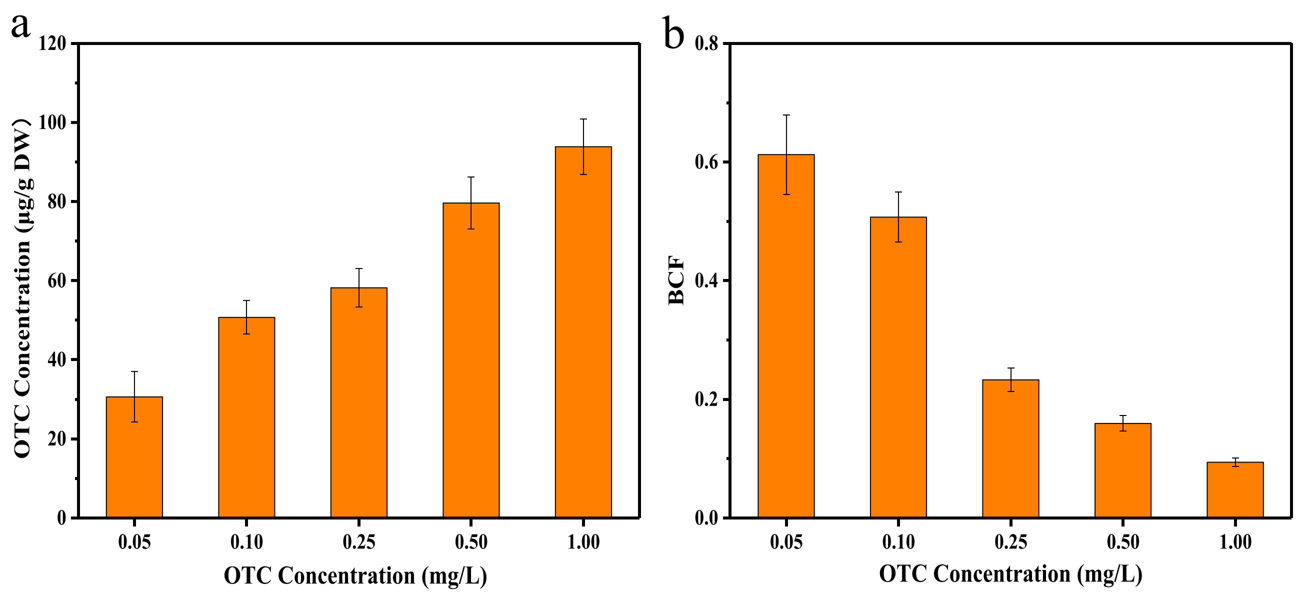

Fig. 4. Oxytetracycline concentrations and bioconcentration factor (BCF) of *Lemna aequinoctialis* after 10 days of exposure.

membrane systems. Therefore, the ability of *Lemna* to consume and transfer antibiotics was limited, and resulted in the BCF was reduced.

3.4. Community-level physiological profile of rhizosphere microorganisms

3.4.1. Effects of OTC on community metabolism

Community-level physiological profile analysis could visualize the differences in community structure and metabolism caused by xenobiotics, and AWCD is the index for evaluating overall activities and catabolic capacities of microorganisms. CLPP analysis of L. *aequinoctialis* RPM under the influence of various levels of OTC was shown in Fig. 5. Originally, the metabolic activities of RPM were quite lower, and the OD values of all groups were no more than 0.1 within 36 h after inoculation. Secondly, the metabolic activities of RPM increased rapidly after 36 h, especially the group with 0.05 mg/L OTC. After 168 h, the metabolic activity of the lowest concentration was much higher than the control. However, the Shannon diversity (H) showed different results (Table S2). According to this phenomenon, it could be hypothesized that antibiotics could decrease the diversity of plants RPM during phytoremediation, which was caused by the inevitable toxicity of antibiotics to microorganisms. Nevertheless, low concentrations of antibiotics couldn't result in a decrease in the metabolic activity of microbes, which may be due to RPM more dynamic and could respond to the stress caused by antibiotics through enhancing metabolism under this situation.

However, the difference was that the metabolic activities of RPM of other treatment groups (0.10, 0.25, 0.50, and 1.00 mg/L) were lower than those in the control group. This may be meant that high concentrations of OTC have two toxic effects on microflora. First is to kill microorganisms or inhibit their reproduction by inhibiting the synthesis of intracellular proteins, and the other is to reduce the metabolism of nutrients source, which further exacerbates the stress attributed to antibiotics. Furthermore, the Shannon diversity (H) of RPM of all OTC treatment groups was significantly lower than those in the control group, but the Shannon evenness (E) was less affected by OTC, and the Richness (R) followed the same trend as Shannon diversity (H) (Table S2). In addition, ciprofloxacin had the same effect on RPM in wetland mesocosms and the soils with the highest sulfachlorpyridazine exposure also showed a reduced metabolic diversity (Schmitt et al., 2004, Weber et al., 2011). Eventually, the presence of antibiotics such as OTC could have reduced the abilities to metabolize carbon-based compounds by microorganisms that may contribute to the reduction of treatment efficiency and increased transport of these compounds through the duckweed ponds entering other water bodies.

Some differences could be observed in the utilization of carbohydrates, amino acids, carboxylic acids, amines/amides, phenolic acids, and polymer between every group (Fig. 5b). Firstly, the carbon source utilization of 0.05 mg/L group was significantly higher in comparison to that of the control group, among which the utilization of amines/amides increased by 143.91% ($p < 0.05$). This corresponded to the previous description and also proved that extreme environments could promote the utilization of carbon sources by microorganisms (Fig. 5a) (Zhao et al., 2018). Then, the inhibition effects of higher concentration of OTC on the utilization of carbon source by L. *aequinoctialis* RPM were slightly higher than the low concentration groups. Thirdly, among the six carbon sources, the utilization of amines/amides was much lower than the other five carbon sources, and the partial treatment groups even couldn't utilize it. In contrast, RPM could make full use of carboxylic acids. This indicated that OTC was more inclined to slaughter RPM which amines/amides is the main carbon source and could increase the metabolism function of microorganisms with carboxylic acids as the main carbon source. In previous studies, the utilization of amines/amides by RPM of saturated constructed wetlands plants in winter also decreased through antibiotics effects, and the utilization of carboxylic acids was higher compared with that (Weber et al., 2011; Zhang et al., 2019). Similarly, Xu et al. (2016) showed that the addition of high sulfadiazine levels significantly decreased the utilization of amines/amides by soil microbial.

3.4.2. Microbial community metabolic profiles

The OD data of six substrates during 96 h of every group was applied to PCA and analyzed to explore the influence of the OTC on microbial CLPP. Two principal components (PC1 and PC2) were extracted which could be used to reflect the metabolic characteristics of the RPM on carbon sources (Fig. 6). Primarily, the eigenvalues of PC1 and PC2 accounted for 96.76% of the observations and means that PC1 and PC2 could reveal the vast majority of the information of all the original variables. Additionally, according to the loading plot, the six substrates illustrated a positive correlation with PC1. Meanwhile, carboxylic acids, polymer, amines/amides, carbohydrates, and amino acids with a high value conduced to the build of PC1, while phenolic acids described a tiny value on the axis of PC1. 2-hydroxybenzoic acid and 4-hydroxyphenyl acid in phenolic acids are secondary metabolites of plants (Seal et al., 2004). Therefore, it was maybe suggested that PC1 could be used to describe the metabolism of RPM to different types of carbon sources in

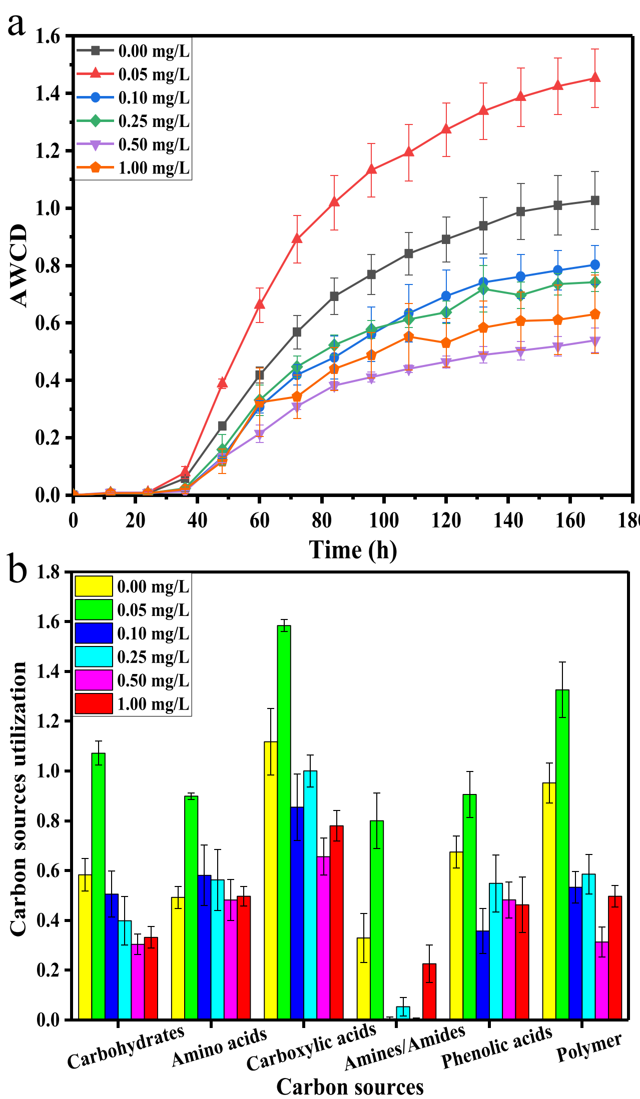

Fig. 5. (a) Metabolic activity of duckweed rhizosphere and phyllosphere microbes under different initial OTC concentrations; (b) Utilization of the carbon source groups by rhizosphere and phyllosphere microorganisms of duckweed at different initial OTC concentrations.

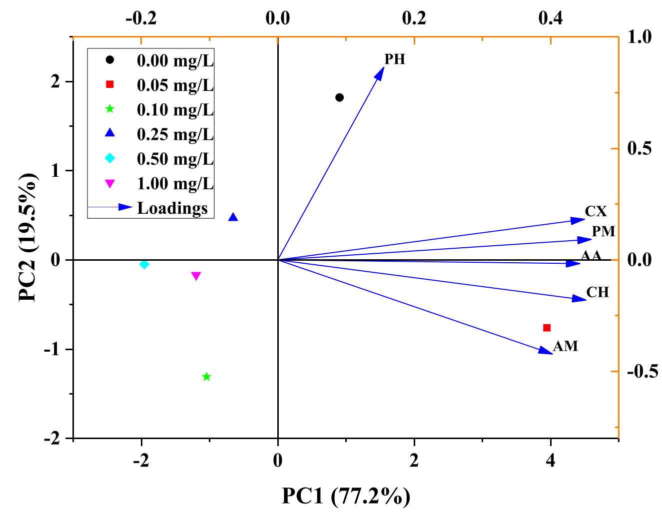

Fig. 6. Principal component analysis (PCA) based carbon source utilization patterns of *Lemna aequinoctialis* rhizosphere microorganisms under different OTC concentration.

this study. The RPM was more likely to utilize the exogenous sources as main carbon sources to structure proteins and maintain the integrity of the cells rather than plant secondary metabolites when the presence of OTC. At last, for the construction of PC2, it was found that the phenolic acids exhibited a forceful positive correlation with PC2, carboxylic acids and polymer showed an infirm positive correlation. Meanwhile, Da Silva et al. (1991) showed that phenolic acids were able to against oxidation by scavenging free radicals and inhibiting the activity of oxidases. Obviously, the phenolic acids can be uptake and metabolize by RPM to enhance their antioxidant capacity, and thus, the PC2 could be used to describe the antioxidant capacities of RPM.

The scores of different OTC level duration of this work also showed in Fig. 6. Firstly, according to the scores plot, it was found that the values of 0.10–1.00 mg/L OTC were negative, while that of 0.00 and 0.05 mg/L OTC were positive in relation to PC1. It showed that the utilization of carbon-based compounds in biolog ECO-plate by duckweed RPM was inhibited at 0.10–1.00 mg/L OTC, but could be fully utilized at low concentration. Subsequently, the scores of the treatment groups with 0.10–1.00 mg/L OTC were closer compared with those in control group and 0.05 mg/L OTC, which indicated that they have the same carbon source utilization patterns. Therefore, high concentrations of OTC could affect the metabolize ability of RPM, indicated that OTC could cause stress on microorganisms by harming their metabolic systems. Furthermore, the control group was to be found in the positive part of PC2, while OTC treatment groups were in the negative part (besides 0.25 mg/L OTC). It was implied that the RPM of the control group with a healthy antioxidant system, which could resist oxidative stress normally. Hence, as a statistical tool, the features of carbon sources utilization patterns and antioxidant capacities of RPM at various OTC concentrations can be further described by PCA.

4. Conclusions

In the initial stage of exposure, OTC could significantly promote the production of H_2O_2 and PODs in duckweed and resulted in duckweed to suffer severe oxidative stress. But after prolonged exposure, duckweed can withstand the stress caused by low levels of OTC (<0.50 mg/L) and restore health.

The synthesis of starches and flavonoids in L. *aequinoctialis* could be enhanced when the OTC concentration in swine wastewater was 0.25 and 0.05 mg/L, and the production of vitamin C was severely inhibited before the exposure is over.

The Michaelis-Menten kinetic model could significantly fit the removal process of OTC by the duckweed systems in swine wastewater. Meanwhile, the bioconcentration study illustrated that the BCF of the 0.05 mg/L group could reach to 0.61.

According to the results of community-level physiological profile, 0.05 mg/L OTC could significantly increase the metabolic activity of duckweed associated microorganisms. The PCA analysis showed that 0.1–1.0 mg/L OTC groups have more similar carbon source utilization characteristics, and antibiotic stress could significantly alter the carbon source utilization mode of duckweed associated microorganisms.

This work might provide a new understanding of the effects caused by antibiotics on high-value nutrients recover from the phytoremediation systems, the interaction between antibiotics and rhizosphere microorganisms and the impact on their metabolic functions. However, the recycle strategies should be mature deliberation and implemented when high concentrations of antibiotics contained in duckweed. Therefore, the sustainable utilization of livestock wastewater could be further improved based on this investigation.

CRediT Authorship contribution Statement

Hao Hu: Conceptualization, Investigation, Methodology, Data curation, Visualization, Formal analysis, Writing - original draft; **Xiang Li**: Conceptualization, Investigation, Formal analysis, Methodology, Writing - review & editing; **Shaohua Wu**: Conceptualization, Investigation, Methodology, Supervision, Writing - review & editing; **Wei Lou**: Methodology, Formal analysis, Writing - review & editing; **Chunping Yang**: Conceptualization, Investigation, Methodology, Writing - review & editing, Supervision, Resources, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.125508.

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